GM-CSF Increases AP-1 DNA Binding and Ref-1 Amounts in Human Alveolar Macrophages

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Alveolar macrophages have been implicated in the pathogenesis of a number of acute and chronic lung disorders. A characteristic feature of many of the chronic lung diseases is that the types of macrophages in the lung change, and in most instances, the cells resemble monocyte-like cells. We have previously shown that normal human alveolar macrophages have a decreased capacity to express protein kinase C (PKC)-induced DNA binding activity of the transcription factor activator protein (AP)-1 compared with monocytes. This decrease in AP-1 DNA binding appears to be due to a defect in redox regulation of AP-1 proteins via a decrease in the redox active protein Ref-1. The hypothesis for this study is that there are factors generated during the development of chronic lung disease that increase AP-1 DNA binding activity and Ref-1 production in human alveolar macrophages. We have focused specifically on granulocyte-macrophage colony-stimulating factor (GM-CSF) as a prototype mediator that can be released by alveolar macrophages and is related to the fibrotic process in the lung. We found that after a 24-h incubation with GM-CSF, AP-1 DNA binding was significantly increased in both unstimulated, interleukin (IL)-13, and phorbol myristate acetate (PMA)-stimulated alveolar macrophages and that there was a corresponding increase in Ref-1 protein by Western blot analysis in the PMA-stimulated group. This suggests that disease-related cytokines such as GM-CSF and IL-13 may modulate AP-1 DNA binding activity in alveolar macrophages.

Alveolar macrophages play a critical role in host defense and in the development of inflammation and fibrosis in the lung. They have been implicated in the pathogenesis of a number of chronic lung disorders, including sarcoidosis, asbestosis, and pulmonary fibrosis (1–3). It has been shown that alveolar macrophages from normal lungs are functionally different compared with those from patients with chronic lung disease. These differences include the spontaneous release of a number of mediators, including cytokines, chemokines, and growth factors for fibroblasts (4–6).

A characteristic feature of many of the chronic lung disorders, including pulmonary fibrosis, is that the types of macrophages in the lung change and become more monocyte-like, both phenotypically and functionally (7, 8). We have shown that normal human alveolar macrophages have a decreased capacity to express protein kinase C (PKC)-induced, mitogen-activated protein kinase activity compared with blood monocytes (9). Additionally, normal alveolar macrophages express very little PKC-induced DNA binding activity of the transcription factor activator protein (AP)-1 as compared with monocytes. This decrease in AP-1 binding activity is due to a defect in redox regulation of AP-1 proteins that occurs secondary to a reduction in the amount of the redox active protein Ref-1 (10). Thus, it is likely that the decrease in Ref-1 in alveolar macrophages plays an important role in maintaining normal alveolar macrophage phenotype and may be modulated during disease states.

AP-1 is a transcription factor composed of homodimers or heterodimers of Fos and Jun family members. These complexes bind to specific DNA sequences in the promoter regulatory region of various genes (11, 12). AP-1 activity is regulated at many levels, including transcription of genes that code for the AP-1 proteins, message stability, and translation of the messenger RNAs (mRNAs). AP-1 activity is also regulated by the composition of the AP-1 complexes, phosphorylation of the proteins and redox regulation of specific cysteine residues in the AP-1 proteins (13–15). The redox status of the AP-1 protein complex is an important determinant of the binding of AP-1 to DNA and is regulated by the nuclear protein Ref-1 (14). This dual function protein acts as both a base excision repair protein and as a redox regulatory protein. The redox function is controlled by thioredoxin, which activates Ref-1 to reduce conserved cysteine residues on Fos and Jun, allowing AP-1 DNA binding to occur (13).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is produced by a number of cell types in the lung. Antigen-stimulated T cells, endotoxin-stimulated monocytes and macrophages, fibroblasts, and endothelial and epithelial cells stimulated by interleukin (IL)-1 or tumor necrosis factor, all release significant amounts of GM-CSF (16, 17). In mice with bleomycin-induced pulmonary fibrosis, GM-CSF is increased in the lung (18). Models of GM-CSF overexpression have also demonstrated the profibrotic nature of GM-CSF. Transgenic expression of murine GM-CSF results in widespread fibrosis (19). Gene transfer studies using adenoviral vectors to overexpress GM-CSF in the rat demonstrate a complex series of cellular and molecular events that ultimately lead to a fibrotic reaction in the lung (20).

IL-13 is a T cell–derived cytokine that has important immunomodulatory and anti-inflammatory properties. It has been shown to be a central mediator in specific T helper (Th)2 pathologies, including asthma and parasitic infections (21). It also appears to play a role in the development of hepatic fibrosis in schistosomiasis infection in mice and is profibrotic in the airways of transgenic mice with targeted pulmonary expression of IL-13 (22).

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Abbreviations: activator protein-1, AP-1; dithiothreitol, DTT; electrophoretic mobility shift assay, EMSA; granulocyte-macrophage colony-stimulating factor, GM-CSF; interleukin, IL; messenger RNA, mRNA; protein kinase C, PKC; phorbol myristate acetate, PMA.

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For this study, we postulate that GM-CSF, which is generated in the lung and is increased during the development of chronic lung disease, together with IL-13, alters Ref-1 amounts and AP-1 DNA binding by alveolar macrophages. Increased AP-1 activity in alveolar macrophages could result in increased release of a number of lung disease–related mediators. To test the hypothesis that Ref-1 amounts can be regulated in human alveolar macrophages, we examined the *in vitro* effect of GM-CSF on these cells. We found that GM-CSF together with IL-13 increases Ref-1 amounts and that this results in increased AP-1 DNA binding.

Materials and Methods

Isolation of Human Alveolar Macrophages

Alveolar macrophages were obtained from bronchoalveolar lavage as previously described (10). Briefly, normal volunteers with a lifetime nonsmoking history, no acute or chronic illness, and no current medications underwent bronchoalveolar lavage. The lavage procedure used five 20-ml aliquots of sterile, warmed saline in each of three segments of the lung. The lavage fluid was filtered through two layers of gauze and centrifuged at 1,500 × *g* for 5 min. The cell pellet was washed twice in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}, and suspended in complete medium, RPMI tissue culture medium (GIBCO BRL, Gaithersberg, MD) with 5% fetal calf serum (Hyclone, Logan, UT) and added gentamicin (80 μg/ml). Differential cell counts were determined using a Wright–Giemsa–stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages. This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

Isolation of Nuclear Extracts and Electrophoretic Mobility Shift Assays

Alveolar macrophages were cultured for 24 h with and without various amounts of GM-CSF. Three hours before harvest, phosphate-buffered saline (control), phorbol myristate acetate (PMA) (100 ng/ml), or IL-13 (10 ng/ml) was added to the cells. The nuclear pellets were prepared by resuspending cells in 0.4 ml of lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid [Hepes], pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid [EDTA]), placing them on ice for 15 min, and then vigorously mixing after the addition of 25 μl of 10% Nonidet P-40. After a 30-s centrifugation (16,000 × *g*, 4°C), the pelleted nuclei were resuspended in 50 μl of extraction buffer (50 mM Hepes, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated on ice for 20 min. Nuclear extracts were stored at −70°C. The DNA binding reaction (electrophoretic mobility shift assay [EMSA]) was done at room temperature in a mixture containing 5 μg of nuclear proteins, 1 μg poly(d(I-C)) and 20,000 cpm of ^{32}P-labeled double-stranded oligonucleotide probe for 30 min. The samples were fractionated through a 5% polyacrylamide gel in 1 × TBE (6.05 g/liter Tris base, 3.06 g/liter boric acid, 0.37 g/liter EDTA-$Na_2 \cdot H_2O$). Sequence of the nucleotide was 5′-CGCTTGATGAGTCAGCCGGAA-3′ (AP-1). Experiments were repeated three times.

Western Blot Analysis

For these studies, alveolar macrophages were cultured for 3 h with or without PMA (100 ng/ml) or GM-CSF. At the end of the culture period, nuclear protein extracts (*see* EMSA protocol) were obtained as previously described (9). The cell material was sonicated for 15 s on ice, allowed to sit for 20 min, and then centrifuged at 15,000 × *g* for 10 min. An aliquot of the supernatant was used to determine protein concentration by the Coomassie blue method. Equal amounts of protein (20 μg for nuclear extracts) were mixed 1:1 with 2× sample buffer, loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel, and run at 80 V for 2 h. Cell proteins were transferred to nitrocellulose (enhanced chemiluminescence [ECL]; Amersham, Arlington Heights, IL) overnight at 30 V and visualized using c-fos–, c-jun–, or Ref-1–specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using a chemiluminescent substrate (ECL; Amersham).

Statistical Analysis

Statistical analysis of the densitometric data was performed by determining the fold increase of all the samples as they relate to the control. Statistical comparisons were performed using a paired *t* test with a probability value of $P < 0.05$ considered to be significant.

Results

GM-CSF Increases Ref-1 Amounts in PMA-Stimulated Human Alveolar Macrophages

To test the hypothesis that Ref-1 amounts can be regulated in alveolar macrophages, we examined Ref-1 protein amounts by Western blot analysis. Alveolar macrophages

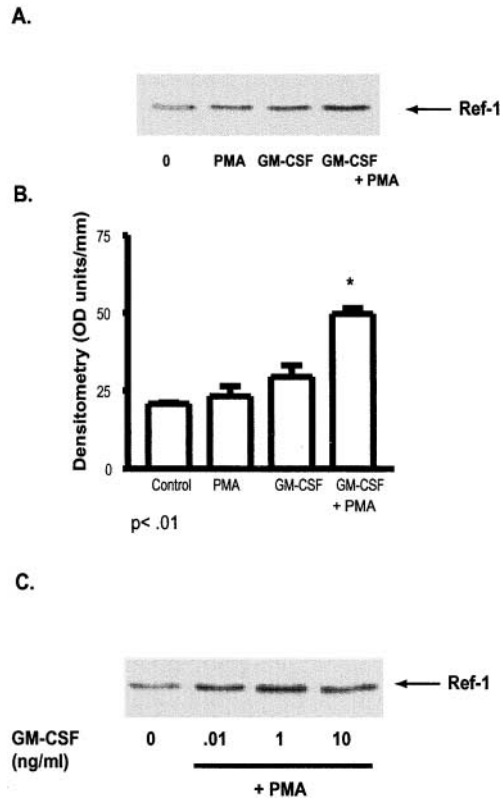

Figure 1. GM-CSF increases Ref-1 amounts in PMA-treated alveolar macrophages. Human alveolar macrophages were cultured for 24 h with and without 10 ng/ml GM-CSF. PMA (100 ng/ml) was added 3 h before harvest and nuclear protein was isolated. (*A* and *B*) Western blot analysis using a polyclonal antibody specific for Ref-1 and the corresponding quantitation of densitometry is shown. The figures illustrate that the addition of PMA following a 24-h incubation in GM-CSF significantly increases Ref-1 amounts in human alveolar macrophages. (*C*) Western blot analysis of nuclear proteins from a similar experiment using varying concentrations of GM-CSF is shown.

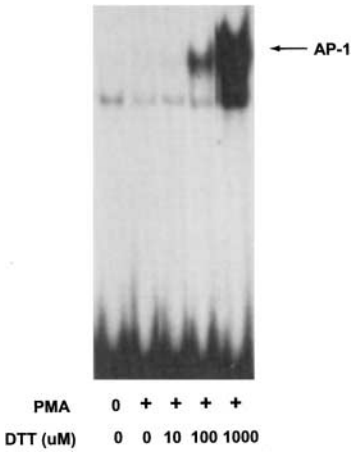

Figure 2. AP-1 DNA binding is dependent on the concentration of exogenous reducing agents. Human alveolar macrophages were treated with 100 ng/ml PMA for 3 h then harvested for nuclear protein. EMSA was performed using the AP-1 consensus oligonucleotide in assay buffer containing varying amounts of DTT. AP-1 DNA binding is enhanced with increasing concentrations of DTT, but does not occur in the absence of reducing agents.

were treated with GM-CSF for 24 h and PMA for 3 h before the cells were harvested for nuclear protein. Western blot analysis was performed using a polyclonal antibody to human Ref-1 proteins. GM-CSF treatment significantly increases Ref-1 amounts when PMA is added 3 h before harvest (Figures 1A and 1B). GM-CSF alone increased Ref-1 amounts above that of the control and PMA-only treated cells; however, this increase was not significant. Additionally, this response appears to be dependent on the dose of GM-CSF (Figure 1C).

AP-1 DNA Binding Is Redox-Mediated in PMA-Stimulated Alveolar Macrophages

DNA binding of the AP-1 protein complex requires that specific cysteine residues be reduced. To confirm the redox sensitivity of AP-1 DNA binding in alveolar macrophages we stimulated cells with PMA, isolated nuclear protein, and performed EMSA using varying concentrations of the reducing agent dithiothreitol (DTT) in the assay buffer. AP-1 binding is dependent on the reducing conditions of the buffer, and increasing amounts of DTT markedly enhance AP-1 DNA binding (Figure 2). In the absence of a sufficient reducing agent, AP-1 binding is abolished. In order to achieve a minimum basal level of AP-1 DNA binding, we used DTT at concentrations of 100 μm in the gel shift incubation buffer.

Effect of GM-CSF on AP-1 DNA Binding in Alveolar Macrophages

To evaluate the effect of GM-CSF on AP-1 binding, we treated alveolar macrophages with GM-CSF for 24 h and harvested cells for nuclear protein. A gel shift assay that

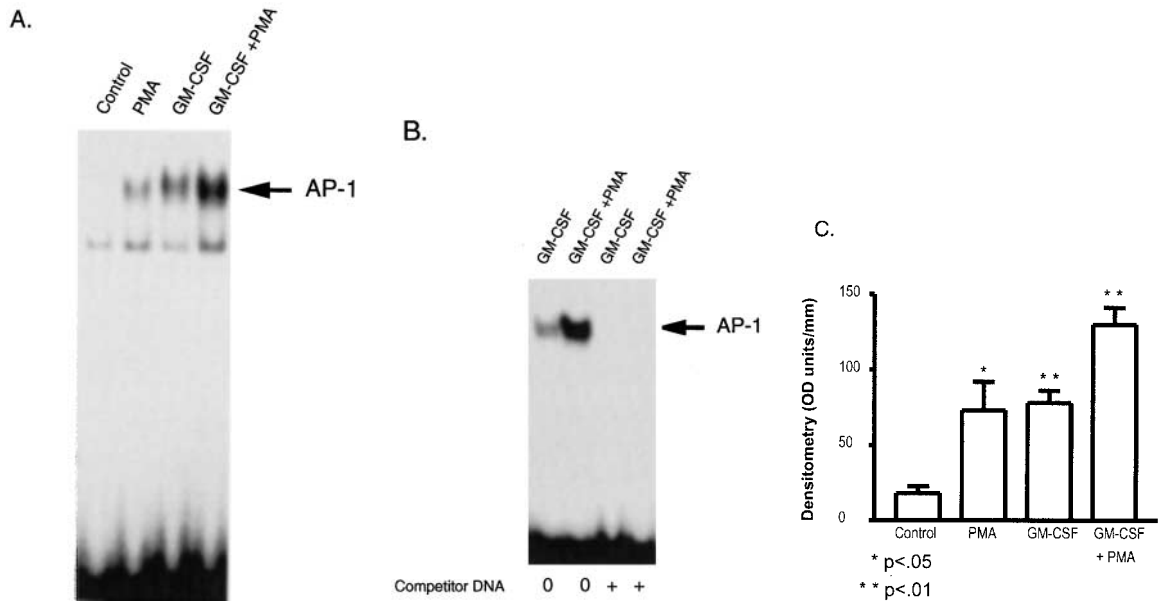

Figure 3. GM-CSF increases AP-1 DNA binding in alveolar macrophages. Human alveolar macrophages were cultured in RPMI medium + 5% fetal calf serum for 24 h at 37°C with and without 10 ng/ml GM-CSF. Three h before harvest, cells were treated with 100 ng/ml PMA or phosphate-buffered saline control. Nuclear protein was isolated, 5 μg of protein was mixed with ^{32}P-labeled DNA, and the protein-bound DNA was separated on a 5% polyacrylamide gel. (*A*) An AP-1 gel shift assay of alveolar macrophage AP-1 binding in cells treated with PMA, GM-CSF alone, or PMA + GM-CSF. The control was also cultured for 24 h. A gel shift similar to *A* was performed using macrophage-derived nuclear proteins with a 100-fold excess of unlabeled AP-1 oligonucleotide added to the reaction mixture. (*B*) The band in the gel shift assay is specific for AP-1 and is completely eliminated by an excess of unlabeled probe. (*C*) A quantitation of the densitometry performed on the gel shift analysis of nuclear proteins from three individuals in separate experiments is shown. Statistical significance was evaluated using the mean gray level values and the Student's *t* test. $P < 0.05$ or < 0.01 as indicated, compared with controls.

shows increased AP-1 DNA binding in cells treated with GM-CSF and with GM-CSF followed by PMA, given 3 h before harvest, is shown in Figure 3A. This band is specific for AP-1, using unlabeled AP-1 oligonucleotide as a competitor in the gel shift assay (Figure 3B). Densitometry from multiple experiments showed a statistically significant increase in AP-1 DNA binding in cells treated with GM-CSF and GM-CSF + PMA (Figure 3C). In separate experiments, macrophages were treated for 24 h with varying amounts of GM-CSF. An increase in AP-1 DNA binding with GM-CSF and GM-CSF + PMA that is dose-dependent is shown in Figure 4.

c-Fos and c-Jun Proteins Are Not Increased in Alveolar Macrophages Treated with GM-CSF

AP-1 DNA binding is regulated on many levels, including production of the proteins composing the AP-1 complex. To evaluate the effect of GM-CSF on the production of the AP-1 proteins c-fos and c-jun, we treated alveolar macrophages with GM-CSF for 24 h, followed by PMA for 3 h and isolated nuclear protein. Western blot analysis was performed using antibodies specific for c-fos and c-jun. These proteins increase with PMA stimulation, but GM-CSF alone does not affect amounts of c-fos and c-jun (Figure 5). Thus, the increase in AP-1 binding with PMA likely is due to an increase in AP-1 proteins, whereas the increase in AP-1 binding with GM-CSF is due to increases in Ref-1. The synergistic effect of PMA and GM-CSF likely is due to the combined increase in Ref-1 and AP-1 proteins.

IL-13 Increases Ref-1 Nuclear Protein and AP-1 DNA Binding When Given as a Co-Stimulator with GM-CSF

To test the hypothesis that stimulation with an additional profibrotic stimulus could further increase Ref-1 amounts in GM-CSF–treated macrophages, cells were treated with GM-CSF for 24 h, with IL-13 added 3 h before harvest. IL-13 together with GM-CSF treatment increases Ref-1 nuclear protein amounts (Figure 6B). Also, macrophages treated with IL-13 alone exhibit increased AP-1 DNA binding as compared with control cells (Figure 6A).

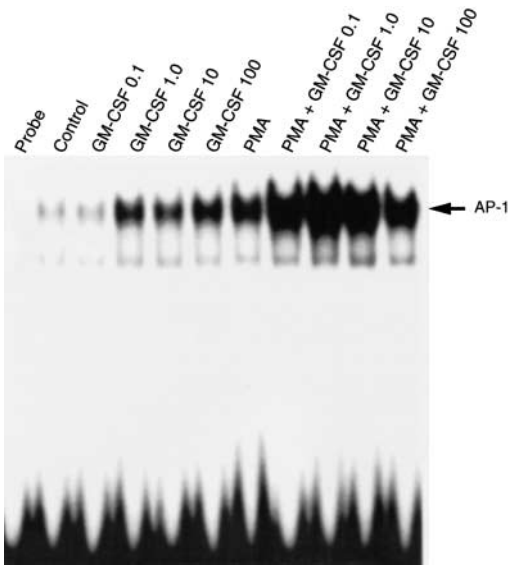

Figure 4. AP-1 DNA binding increases in a dose-response fashion to GM-CSF. Human alveolar macrophages were cultured in RPMI medium + 5% fetal calf serum for 24 h in 0, 0.1, 1.0, 10, and 100 ng/ml GM-CSF. Nuclear protein was isolated, and an AP-1 gel shift assay was performed. This illustrates a concentration-dependent increase in AP-1 DNA binding with GM-CSF.

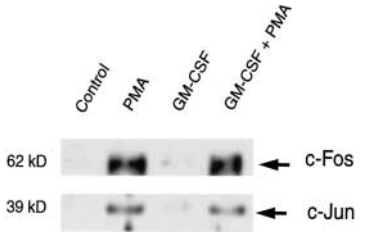

Figure 5. c-Fos and c-jun proteins are not increased by GM-CSF. Human alveolar macrophages were cultured for 24 h with and without 10 ng/ml GM-CSF. PMA was added 3 h before harvest, and nuclear protein was isolated. Western blot analysis of c-jun and c-fos amounts was performed using 10 μg protein per sample. c-Fos and c-jun proteins were visualized using specific antibodies and chemiluminescence. These are autoradiograms of the immunoreactive bands.

Discussion

These studies demonstrate that, *in vitro*, GM-CSF increases AP-1 DNA binding in normal human alveolar macrophages. This effect is further enhanced by the addition of PMA, an activator of PKC, and by IL-13, a T cell–derived cytokine shown to be important in the development of fi-

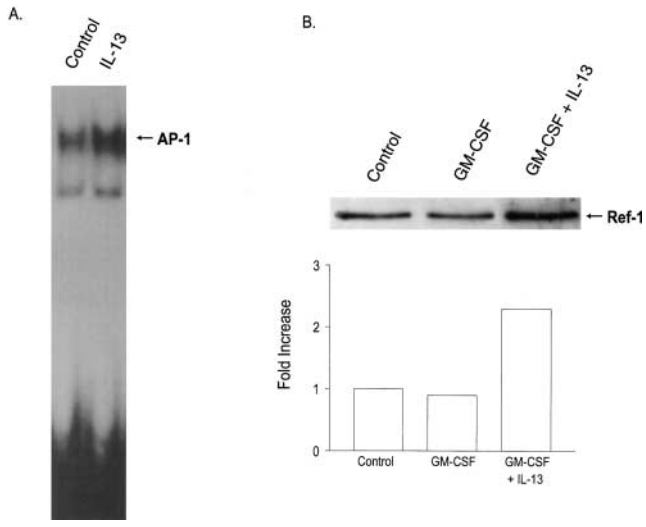

Figure 6. IL-13 increases AP-1 DNA binding and Ref-1 amounts in alveolar macrophages treated with GM-CSF. Human alveolar macrophages were cultured for 24 h with and without 10 ng/ml GM-CSF. IL-13 (10 ng/ml) was added 3 h before harvest, and nuclear protein was isolated. For the gel shift assay, 5 μg of protein was mixed with ^{32}P-labeled DNA, and the protein-bound DNA was separated on a 5% polyacrylamide gel. (*A*) An AP-1 gel shift assay of alveolar macrophage AP-1 binding in cells treated with IL-13. (*B*) A Western blot analysis using a polyclonal antibody specific for Ref-1 and the corresponding quantitation of densitometry. This figure illustrates that the addition of IL-13 after a 24-h incubation in GM-CSF significantly increases Ref-1 amounts in human alveolar macrophages.

brosis in mice. The effect of GM-CSF appears to be due to an increase in the redox active protein Ref-1, whereas the effect of PMA is due to increased amounts of AP-1 proteins. To our knowledge, there are no prior studies that specifically address the question of whether macrophage phenotype could be altered by disease-related factors, other than studies that have previously described the release of mediators from macrophages in response to disease-related stimuli (23, 24). Holian and coworkers (25) demonstrated that asbestos (a model for chronic lung fibrosis) could change the surface phenotype of alveolar macrophages *in vitro*. Our data suggest that normal alveolar macrophages are capable of expressing increased AP-1 DNA binding activity under certain conditions, such as after exposure to GM-CSF and IL-13.

Ref-1 is a ubiquitous, bifunctional nuclear protein that possesses both redox regulatory activity and DNA repair activity. In signal transduction, Ref-1 is important in mediating DNA binding of the AP-1 protein complex (26). This occurs via a post-translational mechanism in which conserved cysteine residues in the DNA binding domains of fos and jun proteins are reduced, allowing DNA binding to occur. We have previously shown that normal alveolar macrophages express decreased AP-1 DNA binding compared with blood monocytes. This appears to be due to a decrease in the amount of Ref-1. Although the functional consequences of this are unknown, we postulate that this may decrease expression of profibrotic genes that are known to be driven by AP-1. AP-1 DNA binding is important for expression of a number of genes whose proteins have been implicated in the development of fibrosis (11, 12, 27), including GM-CSF, alveolar macrophage-derived collagenase (MMP-1), and transforming growth factor-β. We also postulate that certain stimuli, such as the presence of specific cytokines, may trigger AP-1 DNA binding in normal alveolar macrophages, and this may play a role in the initiation/development of fibrosis in the lung. Alveolar macrophages are chronically exposed to cytokines and growth factors during the course of various lung diseases. If some of these factors increase AP-1 activity, it would suggest a mechanism for maintaining AP-1 DNA binding activity in human alveolar macrophages from patients with chronic lung diseases.

GM-CSF has a number of biologic effects, including the maintenance of viability in hematopoetic progenitor cells and mature blood cells, and enhancement of the activity of mature blood cells. In neutrophils, for example, GM-CSF can stimulate an oxidative burst (28). GM-CSF activates transcription of a number of genes and has been shown to increase c-fos and c-jun mRNA in human peripheral blood granulocytes, human myeloid leukemic cells, and peripheral blood (29). To examine the mechanism by which GM-CSF regulates gene transcription, Adunyah and colleagues (29) used a myeloid leukemic cell line (U937) that was transfected with a reporter gene containing an AP-1 driven promotor. They demonstrated that GM-CSF stimulated transcription of the reporter gene and when given to U937 cells and blood monocytes caused an increase in c-fos and c-jun mRNA (29). Furthermore, they showed that the effects of GM-CSF could be abrogated by treating the cells with inhibitors of PKC, suggesting a role for PKC as an intracellular mediator of GM-CSF activity. Thus, both PMA and GM-CSF can increase AP-1 DNA binding, which may in part explain our observations. This, however, does not explain the lack of increased c-fos and c-jun in our studies. One possible explanation is in the functional differences that exist between alveolar macrophages and monocytes. Our group has previously shown that there are substantial differences in PKC isoforms in alveolar macrophages compared with monocytes (9). Alveolar macrophages tend to have fewer Ca^{2+}-dependent isoforms and differential expression of the Ca^{2+}-independent isoforms as compared with monocytes. Also, though there are adequate amounts of c-fos and c-jun mRNA and protein, AP-1 DNA binding is markedly reduced in normal alveolar macrophages. Based on the data presented here, the observation that AP-1 DNA binding in normal alveolar macrophages can be enhanced with GM-CSF does not appear to be due to increases in c-fos or c-jun proteins.

IL-13 is an important immunomodulatory and anti-inflammatory cytokine. It is primarily produced by activated CD4 Th2 cells, but has also been shown to be produced by human alveolar macrophages from patients with pulmonary fibrosis (30). Its effect on primary immune cells is to induce immunoglobulin production, proliferation of B cells, and monocytic differentiation. It is also a potent monocyte chemoattractant. IL-13 has been shown in recent studies to be an important mediator of airway fibrosis in transgenic mice in which IL-13 expression is targeted to the lungs (22). In these animals, collagen was noted to accumulate in the subepithelial and adventitial regions of the airways. It is unknown whether IL-13 is involved in the development of interstitial pulmonary fibrosis. Hancock and associates (30) have shown that human alveolar macrophages from patients with pulmonary fibrosis produce large amounts of IL-13. They postulate that increased production of this cytokine may be involved in the modulation of the inflammatory response that occurs in pulmonary fibrosis and that IL-13 may be produced in response to an effort to downregulate inflammation and induce repair. We have shown that IL-13, when added to cells that are pretreated with GM-CSF, increases amounts of nuclear Ref-1 in human alveolar macrophages. Also, alveolar macrophages treated with IL-13 alone exhibit increased AP-1 DNA binding. The mechanism for these changes is unknown. One possible explanation is that a pro-fibrotic environment in the lung, in which macrophages are exposed to increased amounts of GM-CSF as well as other inflammatory cytokines, IL-13 is produced in an effort to modulate the inflammatory response and enhance repair. This hypothesis has not been tested, but the work of Zhu and coworkers (22) suggests that IL-13 is an important profibrotic cytokine and that chronic expression leads to increased collagen accumulation in the lung.

Only a few prior studies have demonstrated the modulation of Ref-1 expression. Both Ref-1 mRNA and protein levels are increased in mesothelial cells exposed to asbestos (31) and rat thyroid FRTL-5 cells treated with thyrotropin (32). Suzuki and colleagues (33) showed that human chorionic gonadotropin increased Ref-1 in murine Leydig cells. Hypoxic conditions in certain cells lines (34) have also been shown to increase Ref-1 mRNA levels. This

study demonstrates that GM-CSF together with PMA or IL-13 increases Ref-1 protein amounts. Why Ref-1 increases in response to these stimuli is unclear. One possible explanation is that GM-CSF followed by PMA stimulation is altering the redox environment of normal alveolar macrophages, which in turn, is increasing Ref-1 amounts. Diamond and associates (35) have shown that oxidant stress in the form of heat shock in HeLa and NIH 3T3 cells activates the early response genes c-fos and c-jun and that this response is dependent on Ref-1. PMA alone has been shown to generate oxidant stress, which might in turn modulate Ref-1. Prior studies in this laboratory, however, have failed to show increased Ref-1 in normal alveolar macrophages when stimulated with PMA alone. Further studies are needed to examine the role of oxidants in our model of Ref-1 activation.

In conclusion, this study suggests that cytokines such as GM-CSF and IL-13 present during the initiation of fibrosis enhance AP-1 DNA binding and Ref-1 production in normal alveolar macrophages. We postulate that this alteration of normal macrophage phenotype may have important implications in the development of fibrotic lung disease.

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